Iron-Binding Catechols and Virulence in *Escherichia coli*

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Previous work suggested that virulent bacteria, which can grow rapidly in serum, must possess a specific mechanism for removing iron from its transferrin complex. Two strains of *Escherichia coli* were examined with this in mind. Strain O141, which showed inoculum-dependent growth in serum and multiplied in the mouse peritoneum, secreted iron-binding catechols into both synthetic medium and serum. One of these compounds has an association constant for iron similar to that of transferrin. Both transferrin and ethylenediamine-di-o-hydroxyphenyl acetic acid (EDDA), which have very high affinities for ferric iron, induced catechol synthesis in growing cultures of strain O111. This organism was inhibited by normal horse serum. Further work showed that traces of specific antibody inhibited catechol synthesis by O111 exposed to EDDA; therefore, the existence of this inhibitory process means that the organism can no longer obtain Fe^{3+}, which all remains bound to transferrin in serum. In vivo, the inhibition of O111 is similar to that produced by serum in vitro. Neither phagocytosis nor killing by complement appeared to be of any significance during the first 4 h of the infections. Significantly, the purified catechol was capable of abolishing bacteriostasis in vivo. Since these results show that the production of iron-binding catechols is essential for rapid bacterial growth both in vitro and in vivo, these compounds should therefore be considered as true virulence factors. Conversely, any interference by the host with the production or activity of these compounds would constitute an important aspect of antibacterial defense.

There is considerable evidence to show that iron compounds can enhance the growth of bacteria such as *Escherichia coli* (3, 6) and *Listeria monocytogenes* (34) in normal animals, and in both instances the number of organisms required for a lethal infection is greatly reduced. Iron compounds can abolish passive immunity to *Clostridium welchii* (5) and *Pasteurella septica* (10), and can also abolish the bacteriostatic and bactericidal effects of sera against *E. coli* (7), *C. welchii* (30), and *P. septica* (9) in vitro. It was concluded, therefore, that the basis for these antibacterial effects was the ability of transferrin to maintain an extremely low level of ionic iron (approximately 10^{-18} M) in the circulating plasma (9). Transferrin itself inhibits the growth of *C. welchii* in vitro, but the addition of $\beta2$ and gamma globulins reinforces this antibacterial effect (30), and in the case of *P. septica* both specific antibody and complement as well as unsaturated transferrin are required. Bacteria such as *P. septica* (9) and *E. coli* O141 (7), which grow rapidly in normal serum in spite of the low Fe^{3+} concentration, must therefore be able to remove Fe^{3+} from its transferrin complex. This

could clearly be achieved by means of a suitable cell-bound or diffusible chelating agent (9). Such an agent would be essential for bacterial growth in vivo and hence for virulence. Low-molecular-weight iron-chelating compounds have been isolated from pathogenic bacteria. These include hydroxamates from *P. tularensis* (15), mycobactins from *Mycobacterium tuberculosis* (32), and catechol compounds from *Salmonella typhimurium* (27). This communication compares the ability of two strains of *E. coli*, differing in virulence for mice, to produce catechols, and describes the role of these compounds in promoting bacterial growth both in vitro and in vivo.

MATERIALS AND METHODS

Bacteria. Two strains of *E. coli*, O141/K85/H4 and O111/B4/H2, were used (7). The bacteria were collected by centrifugation of 15 ml of a 3-h culture in papain digest broth and were resuspended either in 3 ml of 10% (vol/vol) papain digest broth in saline or in 3 ml of normal saline. An estimate of the bacterial population was then made by nephelometry with the use of standard curves. Viable

counts were made by spreading 0.10-ml volumes of the appropriate dilution on blood agar plates.

Chemicals. Analytical-grade chemicals were used when available. Ferric nitrilotriacetate was prepared at a concentration of 1.0 mM by mixing 1.0 ml of 0.10 M ferric ammonium sulfate with approximately 50 ml of water containing 1.1 equivalents of nitrilotriacetic acid; after adjusting the pH to 7.4 with 1 N NaOH, the volume was made up to 100 ml with water. Ethylenediamine-di-o-hydroxyphenyl acetic acid (EDDA; K & K Laboratories) was freed from contaminating iron before use. A solution containing 10 g in 190 ml of boiling 1 N HCl was prepared; after being cooled and filtered; it was diluted with 1,500 ml of acetone, and the pH was raised to 6.0 by adding 1 N NaOH. After standing overnight at 4 C, the precipitate was filtered off and washed with cold acetone; the yield was 75%. Fresh normal horse serum (no. 3) was obtained from Wellcome Reagents Ltd. and was stored at −20 C.

E. coli antisera. Specific antisera to strains O111 and O141 were prepared in individual horses. The O agglutinating titers were 1:8,000 and 1:20,000, respectively. Serum taken from the horse prior to immunization with strain O111 had an O agglutinating titer of 1:16 against that organism.

Human transferrin. One sample of human transferrin was isolated from acid-citrate-dextrose plasma as previously described (30). A second sample, fully saturated with iron, was obtained from Kabi Pharmaceuticals Ltd. Transferrin was labeled with ^{59}Fe by mixing 20 μCi of the isotope with a few crystals of nitrilotriacetic acid and then adding 0.4 ml of water, 0.4 ml 6% NaHCO₃, 8.0 ml of 0.15 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.4), and finally 1.0 ml of transferrin, which had a total iron-binding capacity of 0.307 mM and was 46% saturated. The mixture was dialyzed against 5 × 100 ml of the Tris buffer before use.

Saturated and unsaturated iron-binding capacities. Iron-binding capacities were determined as described by Bullen et al. (8). Table 1 shows the values obtained for the samples of normal horse serum used throughout these experiments and also for pooled heparinized mouse plasma.

Paper chromatography. Paper chromatography was carried out overnight on Whatman no. 1 paper with 5% ammonium formate plus 0.5% formic acid as solvent (24). The dried papers were examined under ultraviolet light, and the catechols were detected by spraying with an aqueous solution of 1% ferric ammonium citrate plus 1% K₃Fe(CN)₆ and washing in dilute HCl (16).

Radioactive counting. ^{59}Fe was counted with an efficiency of 75% in Triton X-100-toluene (1:2, vol/vol) containing 0.5% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazolyl)benzene (35) with the use of discriminator settings of 200 to 1,000 in a Beckman LS-133 liquid scintillation counter.

Bacterial growth in serum. Horse serum in

TABLE 1. *Iron-binding capacities (micromoles/ liter)*

Determination	Normal horse serum	Normal horse serum	Mouse plasma
Unsaturated iron-binding capacity........	60	40	76
Saturated iron-binding capacity...........	30	63	58
Total iron-binding capacity...........	90	63	134
Percent saturation.....	33	36	43

6-ml amounts was stirred with the aid of magnetic followers in jacketed culture vessels at 37 C supplied with a gas mixture containing 10% O₂-5% CO₂-85% N₂ at 100 ml/min (14). Tests for the bacteriostatic effect of transferrin were carried out in normal saline containing one-quarter strength medium 199, 0.2% NaHCO₃, and 0.1% bovine serum albumin (8) under the same conditions. Samples were diluted 1:10 and then homogenized for 1 min prior to viable counting.

Catechol production by E. coli in synthetic medium. The medium, based on those described by Young et al. (37) and Dvorak and Heppel (13), contained: glucose, 10 g; NH₄OOCCH₃, 2 g; KH₂PO₄, 2 g; Na₂HPO₄, 2 g; NaCl, 0.1 g; MgSO₄· 7H₂O, 50 mg; CaCl₂·6H₂O, 1.0 mg; MnCl₂·4H₂O, 0.5 mg; ZnSO₄·7H₂O, 0.2 mg; CoCl₂·4H₂O, 0.2 mg; and distilled water to 1.0 liter. The iron concentration in the medium was 1.0 μM, as estimated on a 10 times concentrated sample (11). Sterile medium in 900-ml portions was inoculated with 100 ml of bacterial culture in the same medium. The aeration rate was 500 ml/min, and the pH was maintained at 7.5 by automatic addition of 1 N NaOH, by use of a Radiometer titrator type TTTIc. Bacterial growth was estimated by nephelometry. After the cessation of multiplication (6 to 8 h), 60 ml of glacial acetic acid was added, and the bacteria was removed by centrifugation at 670 × g. The supernatant fluid was concentrated to 50 ml by rotary evaporation, and the pH was then adjusted to 2.5 by careful addition of 6 N HCl. The solution was extracted with 3 × 50 ml of ethyl acetate, and the ethyl acetate was washed with 20 ml of 0.1 N HCl and finally dried over Na₂SO₄. A small portion of the oily residue remaining after evaporation of the ethyl acetate was examined by paper chromatography; the remainder was suspended in 8.0 ml of 50% ethanol, the pH of which was raised to 6.5 by careful addition of 5 N NaOH. This sample was tested for its ability to promote bacterial growth by drying a suitable sample in a culture vessel in vacuo and dissolving the residue in 6.0 ml of horse serum. The concentrations under test are expressed in the terms of ratio of the original culture fluid to the serum.

Catechol production by E. coli O141 growing

in serum. The organism was grown from an inoculum of 10^5/ml in 200 ml of horse serum for 16 h at 37 C and pH 7.5, sterile air being passed over the surface of the serum at 200 ml/min. After removing the bacteria by centrifugation for 20 min at $500 \times g$, the pH was lowered to 2.5 by careful addition of concentrated HCl; the serum was then extracted with 3×200 ml of ethyl acetate. The extract was concentrated to dryness, and the residue was dissolved in 20 ml of 10 mM ferric ammonium citrate, pH 5.0, to form the iron catechol complexes, which are insoluble in ethyl acetate. Extraction with 3×20 ml of ethyl acetate then removed a considerable quantity of green pigment. The residue was made 0.1 M in citric acid, and the pH was adjusted to 2.5 to decompose the iron catechol complexes; this solution was extracted with 3×20 ml of ethyl acetate to finally isolate any catechols which might be present. This extract, together with a control obtained by extracting 200 ml of normal horse serum, were concentrated and examined by paper chromatography, after which the remaining material was dissolved in 2.0 ml of 0.15 M Tris-hydrochloride (pH 7.5); 0.60-ml portions were titrated spectrophotometrically at 488 nm in 2.0-cm microcells with 0.01-ml portions of 1.0 mM ferric nitrilotriacetate.

Experimental infections. Theillers original mice of either sex, weighing 23 to 25 g, were used. The bacteria were suspended in 0.2 ml of 10% (vol/vol) broth saline and injected intraperitoneally. Purified iron-binding catechol was dissolved in ethanol at a concentration of 2.0 mg/ml, and a suitable sample was dried in vacuo. The residue was suspended in broth saline to give 0.5 mg/ml. Thus, each animal received 0.1 mg or approximately 4 mg/kg.

LD50 determination. The principle of Reed and Muench (28) was applied, and six groups each containing 10 mice were used. Bacterial suspensions were prepared to cover a range of approximately 1.3 log units. The LD50 was calculated from the death rate at 2 days.

Intraperitoneal bacterial growth. A group of 10 mice was infected, and individuals were killed at hourly intervals with CO_2 gas. A 4.0-ml amount of broth saline was injected into the peritoneum and, after kneading the abdomen for 1 min with blunt forceps, the fluid was removed. The total bacterial count was obtained by homogenizing a 1:10 dilution of the peritoneal washings for 7 min in an ice bath (MSE homogenizer fitted with a 5-ml bottle). Volumes of 0.1 ml of suitable dilutions were spread on blood agar plates to obtain the viable count. A second sample of the peritoneal washings was centrifuged for 5 min at $85 \times g$ to sediment the mouse cells. Viable counts of the supernatant fluid gave a measure of the extracellular bacteria. The accuracy of the method of counting bacteria in the peritoneum was assessed by comparing the viable count in peritoneal washings removed immediately after injection with that calculated from the viable count

of the inoculum. This showed that the number recovered corresponded to approximately 100%.

RESULTS

Growth of E. coli in horse serum. Previous work had shown that *E. coli* O141 exhibited inoculum-dependent growth in normal rabbit serum, whereas *E. coli* O111 was killed under the same conditions by the action of complement (7). *E. coli* O141 also showed inoculum-dependent growth in normal horse serum (Fig. 1), although the lag phase was longer than that found in rabbit serum (7). The addition of 5% specific antiserum reduced the growth rate of the large
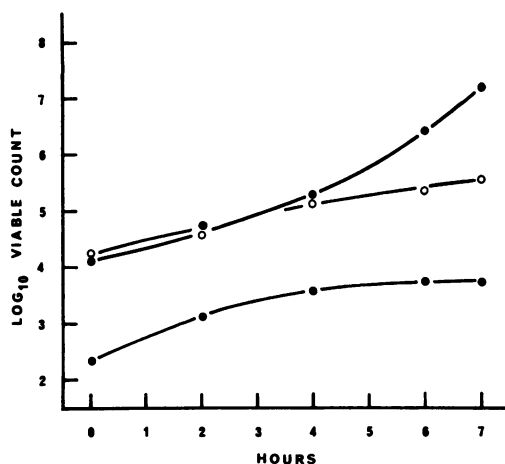

FIG. 1. *Growth curves of E. coli O141:* ●, *normal horse serum;* ○, *normal horse serum containing 5% specific antiserum.*
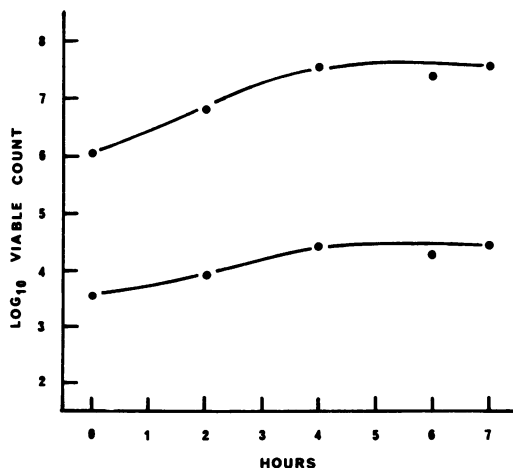

FIG. 2. *Growth curves of E. coli O111 in normal horse serum.*

inoculum. In the case of *E. coli* O111, bacteriostasis was observed after 4 h (Fig. 2).

Effect of transferrin and specific antibody on the growth of E. coli. The growth curves of *E. coli* O111 in one-quarter strength medium 199 containing 15% saturated transferrin (total iron-binding capacity, 20 μM), 0.5% specific antiserum, and a combination of the two are shown in Fig. 3. The mixture induced bacteriostasis after 4 h. On raising the saturation of the transferrin to 100%, the curve became identical to that obtained with antibody alone. Lowering the concentration of antiserum to 0.2% resulted in a loss of the inhibitory effect. Serum obtained from the horse prior to immunization with strain O111, when used at a concentration of 50%, inhibited the growth of strain O111. When mixed at a concentration of 25% with transferrin, the inhibitory effect was lost. Strain O141 grew rapidly from a small inoculum in the presence of transferrin, but the addition of 0.5% specific antiserum led to bacteriostasis for 7 h. With a large inoculum, growth slowly accelerated after 2 h (Fig. 4).

Production of catechols by E. coli. The inoculum-dependent growth of *Bacillus megaterium* in low-iron medium can be overcome by the addition of its own culture fluid, which contains an iron-binding hydroxamate (19). Preliminary experiments showed that 7-h broth culture fluid exerted a similar effect on *E. coli* O141 in serum, suggesting that a product of bacterial metabolism was required for growth in serum. All subsequent work was carried out with
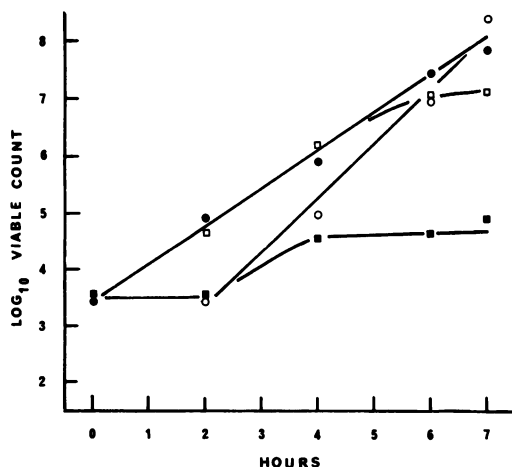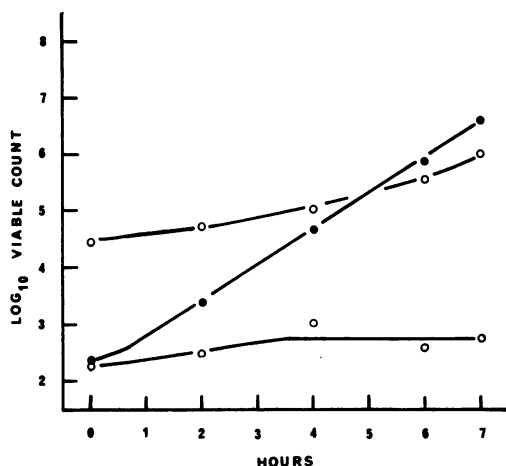

FIG. 4. *Growth curves of E. coli O141 in one-quarter strength medium 199:* ●, *transferrin (total iron-binding capacity,* 20 μM); ○, *transferrin plus 0.5% specific antiserum.*

synthetic medium, which allowed a much easier examination of the culture fluid.

E. coli O141 and O111 grew in the synthetic medium to a maximal population of about 10^8/ml with a generation time of 60 min. Paper chromatography of the ethyl acetate extract of 1 liter of O141 culture filtrate revealed the presence of six catechols having R_f values of 0.18, 0.32, 0.40, 0.53, 0.65, and 0.79 (Fig. 5). No catechols could be detected in similar extracts of culture fluids of O111, but examination of the concentrated extract from a 5-liter culture showed a catechol R_f of 0.75 together with traces having R_f values of 0.18 and 0.53. The extract of O141 stimulated the growth of a small inoculum of O141 in serum (Fig. 6), and the extract of O111 stimulated the growth of O111 in serum. One of the compounds (R_f = 0.53) secreted by *E. coli* O141 has been isolated and tentatively identified (unpublished data) as the methyl ester (Fig. 7) of the acid described by O'Brien and Gibson (26). This compound stimulated rapid growth of a small inoculum of both O141 and *E. coli* O111 in horse serum. It appears that a 0.14 μM solution of this compound in serum may be close to the minimal growth-promoting concentration for O111 (Fig. 8).

Effect of chelating agents on catechol production by E. coli. Although catechol production by *E. coli* appeared to be a constitutive process, it was of some interest to determine whether the addition of competing iron chelating compounds to the medium would change the pattern of catechol production. Extracts of culture fluids obtained by growing *E. coli* O141

FIG. 3. *Growth curves of E. coli O111 in one-quarter strength medium 199:* ●, *transferrin (total iron-binding capacity,* 20 μM); ○, *0.5% specific antiserum;* ■, *transferrin plus 0.5% specific antiserum;* □, *transferrin plus 0.5% normal serum.*
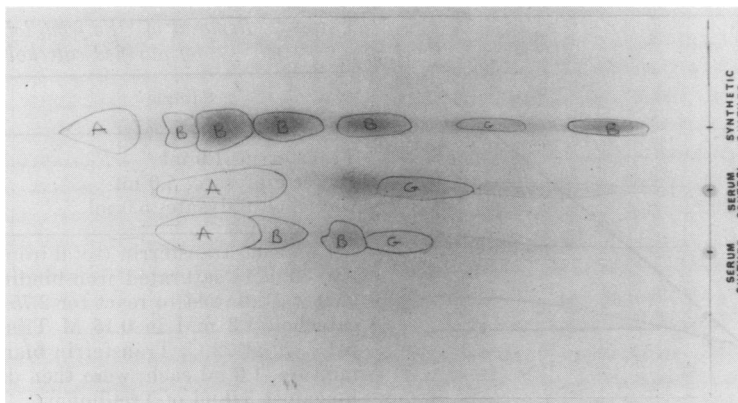
FIG. 5. *Paper chromatogram showing the presence of a catechol compound in the ethyl acetate extract of a serum culture of E. coli O141. The serum control and an extract of a culture grown in synthetic medium are shown for comparison. Letters indicate areas visible under ultraviolet light: A, adsorbtion; B, blue; G, green.*

in the presence of 0.1 mM EDDA contained increased quantities of catechols, particularly those having R_f values of 0.18 and 0.32. Samples of 100 ml were also taken at hourly intervals from a culture in the same medium to examine the sequence of catechol production; no catechols could be detected before 3 h (bacterial population, 2×10^7/ml) when a trace of material with an R_f of 0.18 was visible. One hour later, all six components were present.

Paper chromatography of extracts of *E. coli* O111 culture fluid showed that catechol production was increased by adding 0.1 mM EDDA to the medium. In this case, however, no compound having an R_f of 0.18 was present in the extract. In a further experiment, O111 was grown in 200 ml of one-quarter strength medium 199 containing 15% saturated transferrin (total iron-binding capacity, 20 μM). Two catechols with R_f values of 0.64 and 0.75 were detected in the extract of the 7-h culture.

Since the unsaturated transferrin present in serum is a powerful iron-chelating substance, catechol production by the organism would be required to support growth. Examination of a 16-h serum culture of *E. coli* O141 showed that a small amount of the component with an R_f of 0.65 was present, although this material was absent from the extract of normal serum (Fig. 5). Spectroscopic examination at 488 nm showed that the normal serum extract contained a pink-colored material which was absent from the culture extract. Titration of both samples with ferric nitrilotriacetate produced an increase in optical density (OD) at 488 nm, consistent with the formation of a pink iron complex. The end point of the titration (0.0287 ml) indicated that the catechol had an iron-binding capacity of 0.48 μM in the original serum culture (Fig. 9).
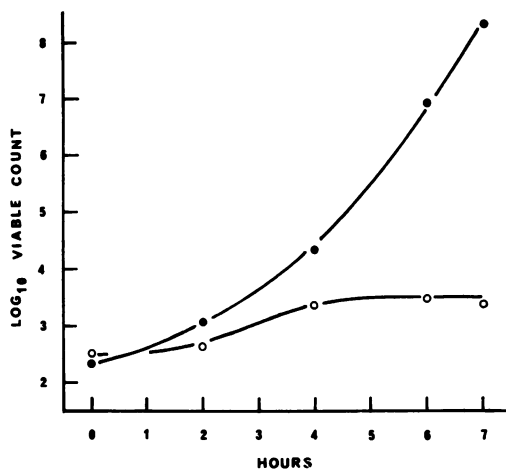

FIG. 6. *Effect of the ethyl acetate extract of E. coli O141 culture fluid on the growth of E. coli O141 in normal horse serum:* ●, *extract equivalent to 30% culture fluid;* ○, *control, no addition.*
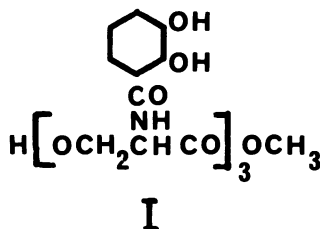

FIG. 7. *Methyl ester secreted by E. coli O141.*

Reaction of the catechol with transferrin. Although the results described above showed that the catechols stimulated bacterial growth in serum, it was essential to demonstrate that they were capable of removing iron from transferrin. These experiments were carried out with the
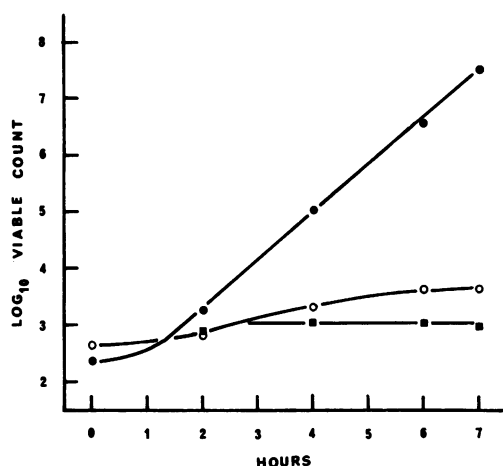
FIG. 8. *Titration of the growth-promoting effect of the purified catechol on E. coli O111 in normal horse serum:* ●, *0.14 μM;* ○, *0.014 μM;* ■, *0.0014 μM.*
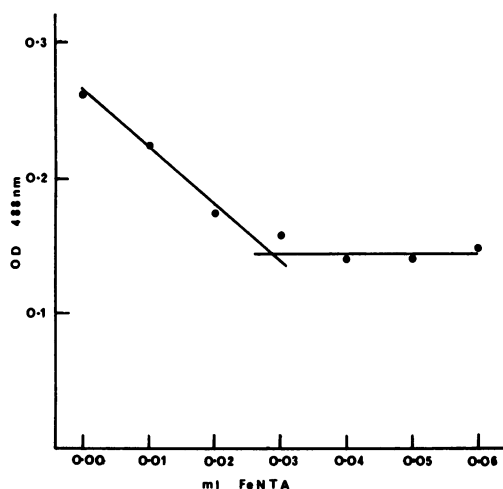

FIG. 9. *Spectrophotometric titration of the catechol isolated from a serum culture of E. coli O141 with 1.0 mM ferric nitrilotriacetate.*

purified catechol, $R_f = 0.53$. Table 2 shows that, with a catechol-transferrin ratio of approximately 50, 25% of the ^{59}Fe label was removed in 5 h.

In a second experiment, 0.09 mM iron-saturated transferrin in 0.15 M Tris-hydrochloride (pH 7.40) with an OD at 470 nm of 0.45 was allowed to react with 1.1 mM catechol at 22 C. After 30 min, it was observed that a pink color was developing, and by 6 h the spectrum had changed to one having λ_{max} at 488 nm with an OD of 0.790 (Fig. 10). This spectrum was almost identical to that calculated for a 0.16 mM solu-

TABLE 2. *Removal of ^{59}Fe from labeled transferrin by purified catechol[a]*

Sample	Total counts/min
Transferrin, 1.0 ml	443,200
Buffer dialysate, 5.0 ml	1,140
Catechol dialysate, 5.0 ml	92,800

[a] Labeled transferrin (total iron-binding capacity, 30 μM; saturated iron-binding capacity, 14 μM) was allowed to react for 2.75 h with purified catechol, 1.3 mM in 0.15 M Tris-hydrochloride, pH 7.40, at 22 C. Transferrin blank and reaction mixture, 1.0 ml each, were then dialyzed for 2.25 h against 5.0 ml of Tris buffer.
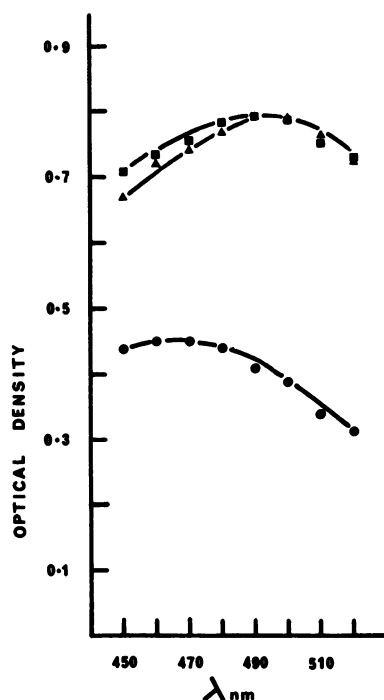

FIG. 10. *Reaction of purified iron-binding catechol with iron-saturated transferrin. Visible spectra of:* ●, *0.09 mM iron-saturated transferrin complex;* ▲, *0.16 mM iron-catechol complex;* ■, *solution 6 h after mixing 0.09 mM iron-saturated transferrin and 0.16 mM iron-binding catechol.*

tion of the synthetic iron catechol complex. The spectrum did not change during the next 12 h, after which the reaction mixture was dialyzed for 64 h against 3.0 ml of Tris buffer at 4 C. The spectrum of the dialysate was identical to that of a 34 μM solution of the iron-catechol complex. The residue had an OD at 488 nm of 0.636, which suggested that some of the complex was non-dialyzable.

By use of the spectrum obtained at 6 h, an approximate equilibrium constant can be calculated for the formal reaction $Fe_2Tr + 2$ catechol $\rightleftharpoons Tr + 2$ catechol Fe. Since $E_{488}^{M} = 4.9 \times 10^3$ for the catechol Fe (unpublished data), it can be calculated that a maximum value of OD at 488 nm of 0.880 would be reached if all of the iron (0.18 mM) had reacted with the catechol. The difference between this value and the observed value of 0.79, i.e., 0.09, corresponds to 18 μM Fe^{3+} still bound to transferrin. Similarly, the relatively high OD value of the reaction mixture in the range from 450 to 480 nm may indicate the presence of some Fe_2Tr. The value of ΔOD at 470 nm of 0.02 corresponds to 8 μM Fe^{3+}. Using the mean of these two figures, 13 μM, we then have $Fe_2Tr = 6.5$ μM, $Tr = 83.5$ μM, catechol Fe $= 167$ μM, and catechol $= 943$ μM.

$$K = \frac{(Tr)\ (Fe\ catechol)^2}{(catechol)^2\ (Fe_2Tr)}$$

$$= 0.4$$

Effect of EDDA and serum on catechol production. Since the high affinity of EDDA for Fe^{3+} stimulated catechol synthesis in both strains of *E. coli*, it was tested at a concentration of 0.1 mM, as a possible replacement for transferrin, with specific antibody in one-quarter strength medium 199. Repeated experiments with O111 failed to detect any inhibition of bacterial growth but, since it was postulated that specific antiserum would inhibit catechol synthesis, ethyl acetate extracts were prepared from 1 liter of culture containing 0.1 mM EDDA and 0.5% of either normal serum or specific antiserum. Paper chromatography showed that a catechol, $R_f = 0.74$, could easily be detected in the extract of the culture containing normal serum, whereas none could be detected in the culture containing antiserum. The more sensitive growth-promoting effect of catechols on *E. coli* O111 in serum was employed to confirm and extend these findings (Table 3). As expected, catechols were present in cultures of O111 containing either 0.5% normal serum or 0.1 mM EDDA but were absent from cultures containing 0.1 mM Fe^{3+} where catechol synthesis would be repressed (38). The addition of 0.5% antiserum almost completely abolished catechol synthesis in the presence of EDDA; this result agrees with that obtained by paper chromatography. Apparently, specific antiserum to O141 was unable to prevent the synthesis of catechols by this organism.

Effect of specific antiserum on the utili-

TABLE 3. *Catechol synthesis by E. coli*

Extract tested[a]	Ratio of culture fluid to serum	Viable count of strain O111
Control (no addition)...	—	1.0×10^4
O111, 0.5% normal serum...	0.5	8.9×10^7
O111, 0.5% normal serum, 0.1 mM Fe^{3+}...	1.0	1.7×10^4
O111, 0.1 mM EDDA...	1.0	2.6×10^7
O111, 0.5% antiserum, 0.1 mM EDDA...	1.0	3.9×10^5
O141, 0.5% antiserum, 0.1 mM EDDA...	0.1	3.6×10^7

[a] The bacteria were grown in one-quarter strength medium 199 containing the substances listed. Ethyl acetate extracts were dried in 1-oz bottles and suspended in 1.0 ml of normal horse serum containing 2.8×10^3 O111 cells to give the ratios shown. Viable counts were made after incubating for 7 h (7).

zation of catechols by *E. coli*. Small inocula of both strains O141 and O111 grew in serum containing 5% specific antiserum and 0.1 μM iron-binding catechol (Fig. 11).

Experiments in vivo. As the results of the experiments presented above showed that catechol synthesis was required for rapid growth in serum, it was decided to compare the virulence, as indicated by LD_{50} measurements, and also the growth patterns in the mouse peritoneum of the organisms.

LD_{50} of E. coli strains. The LD_{50} values were 3×10^6 and 1×10^8 for strains O141 and O111, respectively.

Growth curves of O141 and O111 in the mouse peritoneum. Figure 12 shows the viable counts obtained during intraperitoneal infections with similar doses of the two strains. In the case of O141, where this dose was close to the LD_{100}, bacterial growth commenced after 2 h, and animals began to die at 6 h. In the case of strain O111, the bacterial count remained virtually constant for 4 h, after which a slow decline occurred. Table 4 demonstrates that, during the first 6 h of the infection in normal mice, between 30 and 80% of the bacteria remain in the extracellular fluid. This was confirmed by direct microscopic examination; during one experiment with strain O111 (first column of Table 4), the hemacytometer counts corresponded to 1.8×10^7, 10^7, 2.2×10^7, 2.4×10^7, 0.4×10^7, and 0.8×10^7 extracellular bacteria per mouse at 1 to 6 h, respectively, after infection.
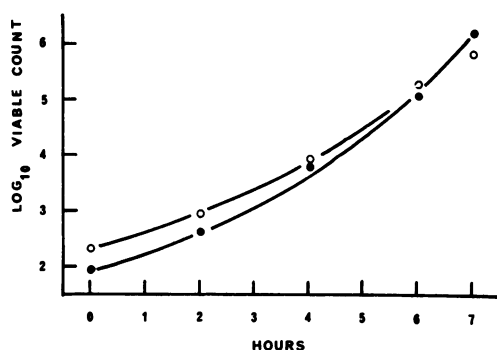
Effect of purified catechol on the intra-

FIG. 11. *Effect of specific antibody on the growth of E. coli strains in normal horse serum containing 0.1 μM iron-binding catechol;* ●, *strain O141;* ○, *strain O111.*
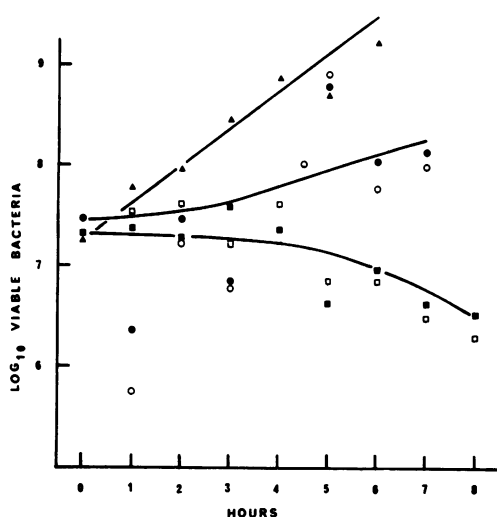

FIG. 12. *Growth of E. coli strains in the mouse peritoneum:* ●, *strain O141 total bacteria;* ○, *strain O141 extracellular bacteria;* ■, *strain O111 total bacteria;* □, *strain O111 extracellular bacteria;* ▲, *strain O111 and purified catechol, 4 mg/kg, total bacteria.*

peritoneal growth of O111. If the failure of growth of *E. coli* O111 in serum were attributed to its inability to produce catechols, the same explanation might account for its failure to grow in the mouse peritoneum. Injection of the purified catechol (4 mg/kg) produced no observable effects in normal mice, but when injected together with O111 the bacteria grew logarithmically (Fig. 12). The animals died within 18 h. This is in marked contrast to the result obtained with 10^7 O111 cells alone, in which case all of the animals survived.

DISCUSSION

The object of this work was to define the role of endogenously produced iron-chelating compounds in promoting the growth of pathogenic bacteria both in vitro and in vivo. It was necessary to demonstrate the production of a well-characterized iron-chelating compound which could promote bacterial growth in serum and also remove Fe^{3+} from its transferrin complex. *E. coli* was chosen for study, as information was already available on its ability to grow in serum (7), and the chemical structure (26) and biosynthesis (12) of the iron-chelating compounds from *E. coli* K-12 have been well characterized. This organism produces five iron-chelating compounds when growing in a low Fe medium. The main component, enterochelin, which is a cyclic trimer of 2:3-dihydroxy-*N*-benzoyl-L-serine, is accompanied by products which appear to arise by cleavage of the ester bonds linking the serine residues (26). Because of the well-known biochemical homologies among the *Enterobacteriaceae*, it would be expected that different strains of *E. coli* would be endowed with similar biochemical pathways for synthesizing catechols. With this in mind, the ability of *E. coli* O141 and O111, which had LD_{50} values of 3×10^6 and 1×10^8, respectively, for mice, have been examined for their abilities both to grow and to produce catechols under a variety of conditions. Strain O141

TABLE 4. *Extracellular bacteria (percent) during intraperitoneal infections of mice with E. coli*

Time (h)	Strain O111			Strain O141		
	2.8×10^{7a}	1.9×10^7	0.9×10^7	4.2×10^7	3.6×10^7	4.1×10^7
1	72	38	67	58	38	57
2	83	115	76	33	63	54
3	30	59	200	53	63	—
4	165	48	44	—	65	79
5	180	35	—	51	30	55
6	74	48	26	76	50	64

[a] Inoculum.

showed inoculum-dependent growth in horse serum similar to that found in rabbit serum (7). With the large inoculum, the increase in growth rate with increase in population could be explained by a parallel increase in availability of the catechols. The addition of 5% antiserum does not completely prevent bacterial multiplication. With O111, an inoculum as high as 10^6/ml is inhibited in normal horse serum (Fig. 1 and 2). Interestingly enough, these results could be simulated by using mixtures of transferrin together with 0.5% antiserum as a source of antibody (Fig. 3 and 4). The results are best interpreted by assuming that both organisms can remove iron from its transferrin complex and that specific antibody prevents this. Complement does not appear to be involved, since previous work had shown that the bacteriostatic effect of both human milk (8) and rabbit serum (7) is heat stable. The fact that only 0.5% specific antiserum when mixed with transferrin exerts a bacteriostatic effect on O111 (Fig. 3) also suggests that complement is not required.

Culture fluids of O141 contained catechols which gave a pattern of spots on paper chromatograms similar to that given by E. coli K-12 (25; Fig. 5). Similar cultures of O111 appeared to contain much lower concentrations of these materials. The ethyl acetate extract of O141 culture fluid stimulated the growth of a small inoculum of O141 in serum (Fig. 6), and the extract of O111 abolished the bacteriostatic effect of serum on O111. The minimal concentration having this effect was approximately 0.1 μM (Fig. 8). This appeared to be a very sensitive method of detecting catechols, since no spot could be detected after chromatography of a 0.1 mM solution of the compound. The fact that the catechols can overcome the bacteriostatic effect of serum on E. coli implies that the synthesis of catechols is required for bacterial growth in serum. A catechol, R_f = 0.65, was found to be present at a concentration of 0.48 μM in a serum culture of O141 (Fig. 5 and 9). The relatively specific iron-chelating compound EDDA, which has an association constant of $10^{33.9}$ for Fe^{3+} (2), enhances catechol production by both organisms, and transferrin enhances catechol production by O111. Brot and Goodwin (4) observed that 2:3-dihydroxybenzoyl serine synthetase activiy appeared in cultures of E. coli K-12 exposed to 2,2'-dipyridyl.

The catechol purified from O141 culture fluid removes ^{59}Fe from labeled transferrin, and the catechol iron complex was identified spectroscopically as a product of the reaction between catechol and iron-saturated transferrin. The equilibrium constant for this reaction appears to

be 0.4, and, since the association constant of the iron-transferrin complex is approximately 10^{36} (1), the association constant for the catechol-iron complex must also be nearly 10^{36}. It may be that the 2,3-dihydroxybenzoyl groups displace the tyrosine hydroxyl groups which are considered to be part of the iron-binding site of the transferrin molecule (1). In agreement with these observations are those of Wilkins and Lankford (36), who showed that 2,3-dihydroxybenzoyl serine stimulated the growth of S. typhimurium in human serum, and those of Kochan et al. (18), who showed that mycobactin overcame the bacteriostatic effect of human serum on M. tuberculosis BCG.

These results suggest that, when present in serum, the catechols transport Fe^{3+} from transferrin to the bacteria, which then multiply. Exposure of the bacteria to chelating agents such as EDDA or transferrin enhances catechol synthesis in response to the low Fe^{3+} concentration. The fact that E. coli O111 does not grow in whole serum therefore suggests that its ability to synthesize or secrete catechols is inhibited under these conditions. With EDDA as an inducer, it was possible to grow both O111 and O141 in the presence of 0.5% serum, as a source of antibody, under conditions which otherwise resembled those used to obtain the growth curves shown in Fig. 2 and 3. The exact mechanism by which the bacteria can obtain Fe^{3+} from its EDDA complex is not known, but it may be that this low-molecular-weight complex can be used directly. Ethyl acetate extracts of these cultures, when tested for catechols by chromatography and their ability to promote the growth of O111 in serum, showed that only in the case of O111 and its specific antibody was catechol synthesis suppressed (Table 3). This is in accord with the observation that bacteriostasis of O111 could only be achieved with its specific antiserum and transferrin (Fig. 3). The results indicate that immunization of the horse with strain O111 results in a 500-fold increase in the O antigen agglutinating titer and a 100-fold increase in the power of the serum to inhibit the growth of strain O111 in the presence of transferrin. From this, it appears that some type of antibody is required for bacteriostasis. A similar antibacterial system appears to operate in human milk, since a mixture of lactoferrin and specific antibody also induces bacteriostasis of O111 (7). The finding that O141 antibody does not inhibit catechol synthesis is in accord with the observation that a large inoculum of O141 multiplies slowly in the presence of specific antibody (Fig. 1 and 4). These results suggest, therefore, that the relative difference in growth of large

inocula of the two organisms in serum is related
to the sensitivity of catechol synthesis by O111
to the inhibitory action of antibody. The factors
which account for these differences are not under-
stood, but the chemical structure and disposi-
tion of the O and K antigens may be important.
The results obtained so far show that the antibody
which acts on O111 must be widely distributed in
nature, since normal rabbit serum (7), cow colos-
trum and human milk (8), and horse serum are all
inhibitory. Since an inoculum of 10^2 O141 cells
per ml can grow in the presence of transferrin but
cannot grow in either normal rabbit serum (7) or
normal horse serum (Fig. 1), it appears that these
sera must contain sufficient antibody to prevent
catechol synthesis by this number of organisms.
It is quite clear, however, that specific antiserum
is unable to prevent the utilization of exogenous
catechols (Fig. 11). The concentration of catechol
employed (0.1 μM) was close to the minimum re-
quired for the rapid growth of O111 (Fig. 8).

The ability of E. coli to grow in vivo has been
considered by Rowley (31) as an indicator of bac-
terial virulence which was related to the capacity
of the organism to resist host defense systems.
The principal defense mechanism in the mouse
has been considered to be phagocytosis by peri-
toneal macrophages (22). A correlation has been
observed between complement-mediated killing
in human or guinea pig serum and susceptibility
to phagocytosis in the mouse (17, 31). These
effects have been related both qualitatively and
quantitatively to the chemical constitution of
the bacterial cell wall. It has been suggested that
the possession of increasing amounts of K antigen
by strains of E. coli is responsible for increased
resistance both to phagocytosis in vivo and to
human complement in vitro (17). E. coli O7 was
resistant to both phagocytosis and guinea pig
complement in vitro and multiplied in the mouse
peritoneum, whereas O111, which was sensitive
to these factors in vitro, failed to multiply in the
mouse peritoneum (22). Two mutants of O111,
with progressively impaired ability to synthesize
O antigen, have been isolated which show a pro-
gressive decrease in mouse virulence (21).

It is, however, doubtful that mouse comple-
ment causes lysis of gram-negative bacteria.
Mouse serum failed to lyse a number of bacteria,
including E. coli, in vitro (20), and no killing of a
variety of bacteria could be detected inside diffu-
sion chambers placed in the peritoneal cavities
of mice (33), although bacterial killing had been
observed by use of the same technique in the
guinea pig (29). It was claimed that complement-
mediated killing of E. coli could be detected in
circulating mouse blood, although similar results
were obtained in complement-deficient mice (23).

Although it is clear that cell wall composition
is one of the factors determining virulence of E.
coli for mice, it appears that insufficient attention
has been paid to the ability of this organism to
grow in vivo, especially when the apparent lack
of bacteriolytic complement is considered.
Another factor is the relatively large number of
bacteria remaining in the extracellular fluid dur-
ing infection of normal mice (Table 4); since the
interaction of these organisms with mouse trans-
ferrin may well determine the outcome of the in-
fection, their behavior in horse serum in vitro
should serve as a guide. Iron compounds abolish
the antibacterial effects of serum on E. coli in
vitro (7) and enhance the virulence of this orga-
nism in both mice (3) and guinea pigs (6). The
mice used in the present experiments had an
average unsaturated iron-binding capacity of 76
μM. There is a striking similarity between the
behavior of large inocula in horse serum (Fig. 1)
and the fact that O141 multiplies in the mouse
peritoneum whereas O111 does not (Fig. 2 and 12).
These differing growth patterns may be reflected
in the relative LD_{50} values. Similar growth pat-
terns were obtained by Medearis and Kenny (22)
with O7 and O111. Neither O141 nor O111 ap-
peared to be readily destroyed by macrophages
in normal mice. This similarity in behavior in
vitro and in vivo is emphasized still further by
the fact that administration of O111 together
with the purified catechol enhanced bacterial
growth sufficiently to convert a nonlethal into a
lethal infection (Fig. 12).

These results support the hypothesis that viru-
lent bacteria which can grow in serum must
possess a specific biochemical mechanism for re-
moving Fe^{3+} from its transferrin complex. This
capability must be considered as a true virulence
factor, since the lack of a suitable defense mecha-
nism by the host would allow the bacterial popu-
lation to reach an insupportable size. One such
case may be P. septica, which grows in normal
serum (9) and is highly virulent for mice, the
lethal dose being fewer than 10 organisms (10).
On the other hand, the ability of the host to inter-
fere with these processes represents an important
aspect of antibacterial defense. The hypothetical
cell-bound iron-transport system in P. septica
can probably be inhibited by antibody, and the
present work shows that specific antiserum pre-
vents the synthesis of diffusible iron-transporting
compounds in E. coli. The behavior of bacteria
which are not phagocytized may therefore be ex-
plained by considering their ability to remove
Fe^{3+} from transferrin and by the capacity of other
serum components to interfere with this process.

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